Evaluation of pulmonary volumetric morphometry at the light and electron microscopy level in several species of passerine birds

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ABSTRACT

The lungs of 3 small passerine species, having similar body mass but different diurnal activity patterns, were analysed morphometrically to assess the relationship between diurnal activity and pulmonary volumetry at the light and electron microscope levels. The percentage volumes of the major lung and exchange tissue components of the 3 species—an aerial insectivore, a foliage gleaner/nectarivore and a ground forager—were strikingly similar, and consistent with literature values for other passerine species. The only significant difference found was exchange tissue plasma volume and pulmonary haematocrit, with the ground-foraging, low activity *Malurus splendens* having significantly lower values than the other 2 species. This may indicate that cardiovascular parameters are more important determinants of metabolic activity in small passerines than aspects of pulmonary anatomy.

Key words: Pulmonary anatomy; avian behaviour.

INTRODUCTION

The last 10 y have seen great advances in the understanding of structure and function of the avian lung, especially from the application of the development of stereological techniques. Studies in this field have led to a greater understanding of how birds achieve such high levels of pulmonary oxygen exchange despite the restrictions on lung volume and lung mass imposed by flight.

The lungs of passerine species appear to be particularly well adapted for a high rate of oxygen exchange, having a significantly larger volume of exchange tissue and a significantly thinner blood–gas barrier than nonpasserine species (Maina, 1984; Maina et al. 1989). It is well known that passerine birds have a higher metabolic rate than nonpasserine birds (e.g. Lasiewski & Dawson, 1967; Aschoff & Pohl, 1970). Thus these anatomical features of passerine lung are presumably a functional reflection of the higher metabolic rates of passerine species.

Past studies have attempted to relate differences in pulmonary morphometry to the activity of birds from different avian orders. However, few studies have minimised the impact of phenotypic divergence on data by investigating more closely related species. In the current study, pulmonary volumetry was quantified stereologically for 3 species of passerine with different metabolic activity levels. The aims of the study were (1) to investigate the relationship between pulmonary morphometry and metabolic activity within the most metabolically active orders of birds, the *Passeriformes*, and (2) to compare methodologies and data with existing studies of pulmonary morphometry in passerine species.

MATERIALS AND METHODS

Species

Three species of passerine were examined in this study. The welcome swallow, *Hirundo neoxena*, is an aerial forager, like all hirundine species. The energy requirements of aerial foraging may be 16–38 % more than that of similarly sized, nonaerial foraging birds; species employing this foraging technique have been found to have significantly higher field metabolic rates

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than nonaerial foragers (Williams, 1988). Consequently, *Hirundo neoxena* was selected to represent high activity passerines.

The brown honeyeater, *Lichmera indistincta*, is nectarivorous and insectivorous. This species does not engage extensively in aerial foraging, although it does fly for 11–14% of daylight hours (Collins et al. 1980; Vitali, personal observations). Nectar acquisition and foliage gleaning require less energy expenditure than aerial foraging (Pyke, 1980; Goldstein, 1988). Because it has a mix of high and low activity behaviours, *L. indistincta* was classed as a moderately active passerine.

The splendid wren, *Malurus splendens*, forages almost exclusively at ground level, hopping in the understorey of its well demarcated territory (Russell & Rowley, 1993). Malurids are rarely observed in foliage more than 1 m above the ground (Wooller & Calver, 1981; Recher et al. 1985; Rowley & Russell, 1990) and have limited capacity for sustained flight, rarely covering more than 30–40 m at a time (Hutton, 1991). *Malurus splendens* was classified as a low activity passerine.

Capture and collection of specimens

Birds used in the study were collected under licence from the Fitzgerald River National Park (34° 12′ S, 119° 24′ E) and the Murdoch University campus, (31° 57′ S, 115° 52′ E), both in the state of Western Australia. Birds were collected by mist netting, and for each species, 6 adult plumage individuals (male or female) were studied. The sex of each individual was determined on dissection (Table 2). All individuals were healthy at the time of euthanasia.

Euthanasia and lung tissue preparation

Pentobarbitone sodium was administered orally until a state of deep anaesthesia was induced. Birds were weighed to an accuracy of ± 0.01 g (Table 2). The trachea was cannulated, and half strength Karnovsky's fixative (Casotti, 1993) was perfused into the lungs in situ at a constant 25 cm head of pressure.

The ribs and lungs were dissected from the rest of the body and placed in a vacuum chamber for 1–2 h to facilitate fixative penetration. The lungs were then dissected carefully from their attachments to the ribs, before returning them to a vacuum chamber overnight to ensure complete tissue fixation. To separate the left and right lungs, the primary bronchi were severed as close as possible to their entry point into the lung parenchyma. Each lung was trimmed to remove all visible extrapulmonary tissue.

The displacement method described by Scherle (1970) was used to estimate the volume of each lung. A paired t test showed that there was no significant difference between the displacement volumes of the left and right lungs of individual birds ($P > 0.05$). The left lung from each bird was transferred to 10% neutral buffered formalin for light microscopy, and the right lung to Sorensen's 0.01 M phosphate buffer for electron microscopy.

Light microscopy

After processing through a series of graded alcohols (70–100%) up to chloroform, the lung was infiltrated with paraffin wax. The lungs were oriented at embedding so that the cranial pole was cut first. The position of the first section was made at random within a predetermined distance 't' from the cranial pole. Transverse parallel interrupted serial 5 μm sections were taken from the lung, the distance between consecutive levels being set at 't'. The magnitude of 't' varied between species, being 1/10 of the mean lung length for each species. Consequently, each lung was represented by 9–11 tissue sections. Sections were mounted on slides and stained with haematoxylin and eosin (Fig. 1*a*).

A series of computer-generated stereology test systems (Grid, Graffiti Data Corporation, Denmark) were superimposed onto a monitor image of slides mounted on a microscope. The image was viewed at a magnification of $\times 250$. The randomised subsampling procedure described by Weibel (1970) was used to scan slides for point counting. The total point count was 1000–2000 per lung. Total point counts of this magnitude resulted in a coefficient of error of less than 7% for all parameters except the primary bronchus, which was not of functional significance to this study. For a full description of the point counting technique, see Gundersen et al. (1988).

Four lung components were identified for point counting at the light microscopy level. These were: lumina of secondary bronchi and parabronchi, exchange tissue, blood vessels larger than capillaries, and other pulmonary tissue. The atria, which evaginate directly from the parabronchus, were classified as parabronchial lumen, as they have been reported to have little role in gas exchange (Duncker, 1972). However, the infundibula, which branch off the atria (usually 1–2 per atrium), were included as part of the exchange tissue. Atria were distinguished from infundibula by their direct luminal evagination from the parabronchi, as well as by the smooth muscle associated with their walls.

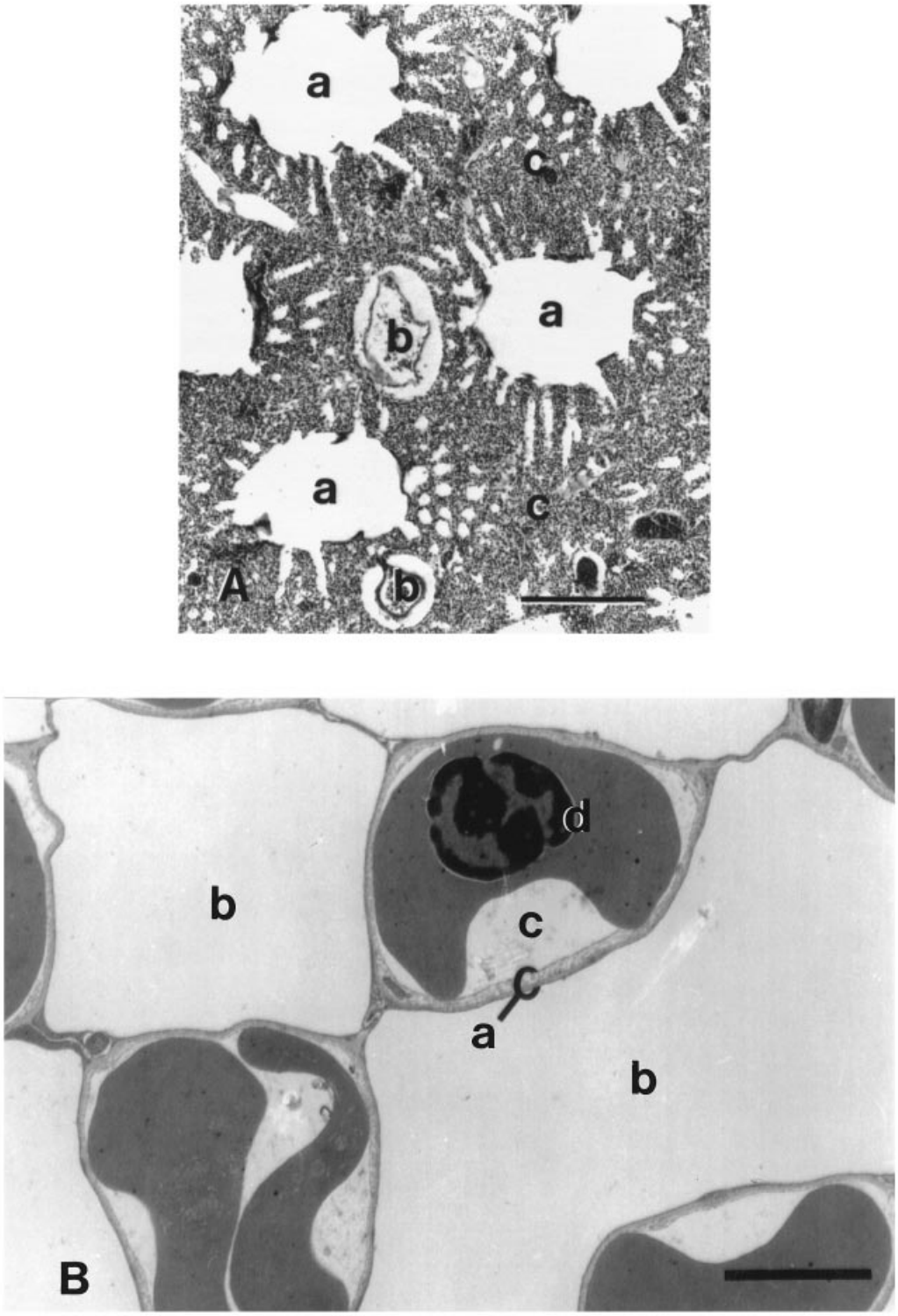

Fig. 1. (*a*) Light micrograph of the lung of *L. indistincta*. a, parabronchial lumen; b, blood vessel larger than a capillary; c, exchange tissue. Bar, 200 μm. (*b*) Transmission electron micrograph of the lung of *H. neoxena*. a, tissue barrier; b, air capillary; c, plasma in blood capillary; d, erythrocyte in blood capillary. Bar, 2 μm.

The intrapulmonary primary bronchus, walls of the bronchi and parabronchi, blood vessel walls, and atrial muscle were not of functional significance to this study, and consequently were grouped together as 'other pulmonary tissue'.

Histological sections frequently contained a small amount of 'nonpulmonary' tissue, such as gut, adipose, extrapulmonary connective tissue, muscle or extrapulmonary primary bronchus. This volume was quantified, and deducted from the original displace-

ment volume, thereby allowing the actual volume of the lung to be estimated.

Electron microscopy

Prior to sectioning, the right lung was embedded in 5% agar to make it easier to cut without distortion. The lung was sectioned, parallel to its costal sulci, into 8 slabs. Each slab was laid flat, then sectioned into a number of segments, 2–3 mm wide. One segment was selected at random from each slab, giving a total of 8 per bird. The selected segments were cut into 1 mm cubes. One cube from each segment was selected at random for TEM processing, yielding a total of 8 tissue samples per bird.

The selected tissue cubes were fixed in osmium tetroxide, dehydrated through a series of graded (70–100%) alcohol washes, cleared in propylene oxide, infiltrated with propylene oxide/Epon mix (60:40), and finally infiltrated with pure Epon resin overnight.

The stereological techniques implemented in this study require the generation of isotropic uniform random (IUR) sections. However, IUR orientation is difficult, if not impossible, to obtain in the small pieces of tissue used in electron microscopy. This problem was solved by implementing the 'isector' technique (Nyengaard & Gundersen, 1992): a spherical shape was imparted to the tissue samples by embedding each one in a sphere of Epon. Isotropy was achieved by rolling the spheres on a flat surface before embedding them in the usual fashion.

Ultrathin sections were cut from each tissue sample and mounted on 200 mesh copper grids. The grids were counterstained with lead citrate and uranyl acetate and then carbon coated for viewing in a Philips 301 transmission electron microscope. Three micrographs were taken from the top left hand corner of consecutive grid squares for each sample, yielding a total of 24 micrographs per bird for analysis (Fig. 1*b*). Final print magnification was $\sim \times 10000$.

A test system of points was superimposed on each micrograph. Point counts were made of 4 components: lumina of the air capillaries; lumina of the blood capillaries (which contained plasma and erythrocytes); tissue barrier; and tissue not involved in gas exchange. The volume of each component was calculated from these point counts as described by Weibel (1970/71).

Any air space which did not communicate directly with the parabronchial lumen was classified as air capillary. This excluded the atria, which were distinguished from air capillaries by their associated musculature, and their direct evagination from the parabronchial wall. Air capillaries were frequently observed to be in direct contact with one another, as were blood capillaries. The places where 2 air capillaries or 2 blood capillaries contact one another are not blood-gas interfaces, and therefore were classified as tissue not involved in gas exchange.

Table 1. *Pulmonary stereological parameters investigated and abbreviations used**

V_L	Volume of lung
V_X	Volume of exchange tissue
$V_{s/p}$	Volume of secondary and parabronchi
V_{bv}	Volume of blood vessels larger than capillaries
V_o	Volume of other pulmonary tissue
V_t	Volume of tissue barrier
V_a	Volume of air capillaries
V_c	Volume of blood capillaries
V_e	Volume of capillary erythrocytes
V_p	Volume of plasma
V_{tn}	Volume of tissue not involved in gas exchange
Ht_s	Stereologically derived pulmonary haematocrit

* Volumes are expressed in mm^3.

Table 1 shows the abbreviations of the pulmonary stereological parameters under investigation. These will be used throughout the text.

Statistics

All analyses were performed on log transformed data. Multivariate analysis of variance (MANOVA) was performed to assess the effect of species and sex on means. The effect of sex was not significant for any of the dependent variables and was therefore eliminated as a dependent variable before analysis of covariance (ANCOVA).

Log transformed data were regressed to the appropriate size covariate (body mass, lung volume or exchange tissue volume) using MANOVA. The size covariate selected depended on the variable under analysis; for example, the volumes of the major lung components were regressed to lung volume, whereas the volumes of the components of the exchange tissue were regressed to exchange tissue volume. Where more than one variable showed a significant regression with the data in question, detrended q–q analysis of residuals was used to determine the most robust independent variable to use for size corrections.

The individual species regressions of log transformed data to the selected size covariate were tested for a common slope using ANCOVA. All regressions demonstrated common slopes across species.

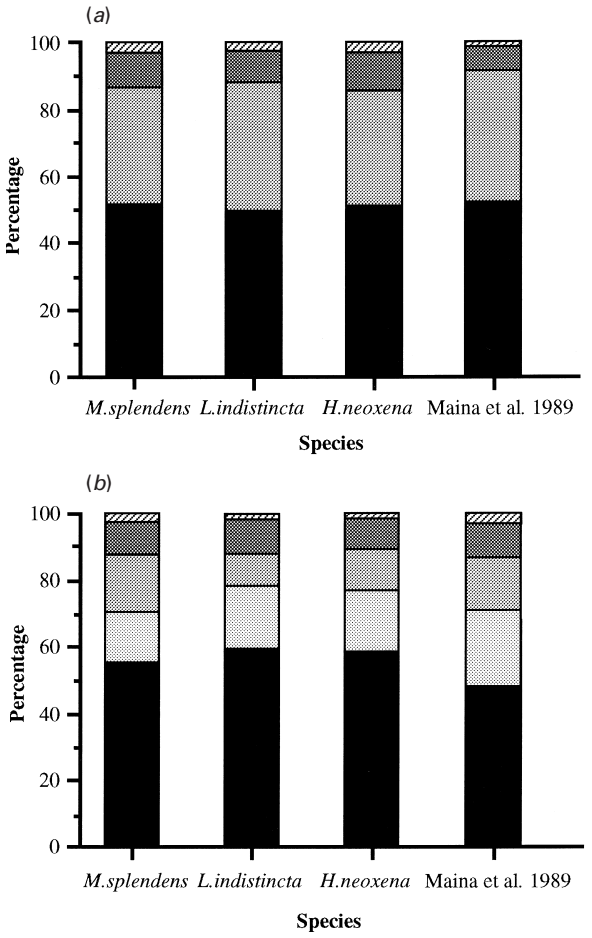

Fig. 2. (*a*) Mean percentage volumes of major lung components for passerine species in the present study and in Maina et al. (1989). ■ = exchange tissue; ⊡ = secondary bronchi and parabronchi; ▩ = blood vessels larger than capillaries; ▨ = other pulmonary tissue. (*b*) Mean percentage volumes of pulmonary exchange tissue components for passerine species in the present study and in Maina et al. (1989). ■ = air capillaries; ⊡ = erythrocytes; □ = plasma; ▩ = tissue barrier; ▨ = tissue not involved in gas exchange.

The residuals from the regressions were calculated to correct for differences in body mass between individuals. Differences between the mean residuals for each species were analysed using the Student–Neuman–Keuls multiple range test.

Raw percentage data, as illustrated by the frequency histograms in Figure 2, were arcsine transformed before statistical analysis to ensure normality of distribution (Zar, 1984). The arcsine transformed data were analysed using the Student–Neuman–Keuls multiple range test.

The mean percentage volumes of each parameter for the study species were compared with values cited by Maina et al. (1989). The values from the current study were considered to be significantly different from those of Maina et al. (1989) if they differed from the latter values by more than 3 standard deviations. A 95% confidence interval was applied to all statistical analyses.

RESULTS

The regression of lung volume (V_L) to body mass was highly significant. The exponent of the regression was 0.54. Both *L. indistincta* and *M. splendens* had significantly smaller V_L values than *H. neoxena*; *Malurus splendens* had a significantly lower V_L than the other 2 species (Table 2).

The absolute volumes (mm^3) of the major lung components (secondary and parabronchi, exchange tissue, blood vessels larger than capillaries, and other pulmonary tissue) were significantly different between species (Table 3). However, when corrected for lung volume, the volumes of the major lung components were not significantly different across species and activity categories.

Figure 2*a* illustrates the percentage volumes of the major lung components for each species. The exchange tissue occupied the majority of the total lung volume in all species (49.7–51.3%), followed by secondary and parabronchi (34.2–38.2%) and blood vessels larger than capillaries (9.7–11.5%). Other pulmonary tissue comprised 2.3–3.1% of the total lung volume. There were no significant differences between the 3 species with respect to the percentage volumes of the major lung components. Figure 2*a* also illustrates the mean percentage volumes of the major lung components as calculated by Maina et al. (1989) for 21 passerine species. In the latter study, the mean percentage volumes of the 4 major lung components—exchange tissue, secondary and parabronchi, blood vessels and other pulmonary tissue—were 52.2%, 39.0%, 7.4% and 1.4% respectively. The percentage volumes of the major lung components in the present study were not significantly different from the results of Maina et al. (1989).

The unadjusted volumes (mm^3) of the exchange tissue components (air capillaries, tissue barrier, plasma, erythrocytes, and tissue not involved in gas

Table 2. *Body mass, lung volume and sex ratio in 3 passerine species; values are means ± S.E.M.*

Species	n	Body mass (g)	Lung volume (mm^3)	Sex (M:F)
Malurus splendens	6	9.68 ± 0.41	231.92 ± 10.32	1:1
Lichmera indistincta	6	9.58 ± 0.48	361.14 ± 22.11	1:2
Hirundo neoxena	6	13.33 ± 0.17	541.13 ± 23.82	1:1

Table 3. *Mean* (±S.E.M.) *percentage and absolute volumes of the major components of the lung in 3 passerine species*

	M. splendens		*L. indistincta*		*H. neoxena*	
	%	mm^3	%	mm^3	%	mm^3
V_x	51.3±1.1	119.1±6.3	49.7±1.6	179.9±14.1	51.3±1.5	276.9±12.6
$V_{s/p}$	35.3±3.7	81.8±4.7	38.2±3.8	137.5±7.9	34.2±2.9	185.7±12.1
V_{bv}	10.3±0.3	23.8±1.2	9.7±0.6	35.3±3.9	1.5±0.2	62.1±2.9
V_o	3.1±0.6	7.2±1.7	2.3±0.4	7.7±2.0	3.0±0.5	15.9±3.6

Table 4. *Mean* (±S.E.M.) *percentage and absolute volumes of the components of the pulmonary exchange tissue in 3 passerine species*

	M. splendens		*L. indistincta*		*H. neoxena*	
	%	mm_3	%	mm^3	%	mm^3
V_a	55.2±2.9	65.3±3.7	59.5±2.3	106.7±8.6	58.4±2.2	161.1±7.1
V_t	10.1±0.9	12.2±1.5	10.1±0.4	18.1±1.6	9.1±0.6	25.4±2.1
V_p	16.7±2.0	19.9±2.7	10.0±0.6	17.9±1.8	12.2±1.0	34.2±3.7
V_e	15.4±2.1	18.7±3.2	18.9±1.5	34.4±5.0	18.3±0.9	51.0±4.2
V_{tn}	2.5±0.6	2.9±0.8	1.6±0.3	2.8±0.4	1.9±0.3	5.2±0.9
$V_e:[V_e+V_p]$	0.48±0.05		0.65±0.01		0.60±0.02	

exchange) were significantly different across species (Table 4). However, when the data were corrected for exchange tissue volume, the only component which differed significantly between species was V_p, the plasma volume: *Malurus splendens* had a significantly higher total plasma volume than *L. indistincta*, but the plasma volume of *M. splendens* was not significantly different from that of *H. neoxena*. Table 4 also shows mean volumes of the exchange tissue components as percentages of exchange tissue volume. The majority of the exchange tissue consisted of air capillary lumina (55.2–59.5%; Table 4), followed by erythrocytes (15.4–18.9%), plasma (10.0–16.7%), tissue barrier (9.1–10.1%) and tissue not involved in gas exchange (1.6–2.5%). Blood capillary volume (the sum of erythrocyte and plasma volumes) constituted 28.8–32.1% of the exchange tissue volume. *Malurus splendens* had a significantly higher percentage plasma volume than the other 2 species, which were statistically indistinguishable. The percentage volumes of all other exchange tissue components were not significantly different across species and activity categories.

Figure 2*b* summarises the mean percentage volumes of the major exchange tissue components for the 3 species, alongside the corresponding values from Maina et al. (1989), based on data from 10 passerine species. The percentage volumes of the air capillaries, erythrocytes, plasma, tissue barrier and tissue not involved in gas exchange were 48.5%, 22.6%, 15.8%, 10.1% and 3.0% respectively for the study by Maina et al. (1989). The mean percentage volume of the air capillaries in the species in the present study were significantly higher than in the study by Maina et al. (1989). No other exchange tissue component volumes differed significantly between the 2 studies.

The sum of the erythrocyte and plasma volumes (V_e+V_p) is equal to the total volume of the blood capillary lumina, V_c. The ratio of erythrocyte volume to blood capillary volume (V_e/V_c) is a stereologically derived estimate of the haematocrit of the pulmonary circulation (Ht_s; Maina et al. 1989). The value of Ht_s ranged from 0.48 in *M. splendens* to 0.65 in *L. indistincta* (Table 4). *Malurus splendens* had a significantly lower stereologically derived pulmonary haematocrit than the other 2 species.

DISCUSSION

The data for stereologically derived pulmonary volumetry in the 3 passerine species of the present study are consistent with the most recent literature data. The only significant difference was the higher percentage volume of air capillaries (V_a) in the present study than for the passerine species studied by Maina et al. (1989). It is possible that this reflects methodological differences, as Maina et al. (1989) did not

explain how they distinguished infundibula from air capillaries at the TEM level; if they did not include infundibula as part of the exchange tissue, then air capillaries would occupy a relatively smaller percentage of the total exchange tissue. Nevertheless, further study of air capillary volume is warranted to assess whether the difference between the species of Maina et al. (1989) and those of the present study are biological or methodological.

Differences in pulmonary stereology between closely related avian species are discernible even at the light microscopy level. Vidyadaran (1987) showed that the exchange tissue volume of the domestic chicken, *G. gallus var domesticus*, was significantly lower than that of the red jungle fowl, *G. gallus*. Maina (1987) suggested that amongst charadriiform species, those which forage by diving have a larger proportion of blood vessels larger than capillaries (V_{bv}) than those that forage by gliding flight. Other studies (Maina, 1982; Maina et al. 1989) reported that the high proportion of pulmonary exchange tissue (V_x) found in passerine species is associated with their high metabolic rates and field energetics.

Such studies suggest that there is a relationship between field energetic demand and the volume of pulmonary and exchange tissue components. We would therefore expect the more energetic *H. neoxena* to have the highest exchange tissue volume (V_x) of the species under study, reflecting the increased requirement for rapid oxygen delivery in this species, and *M. splendens* to have the lowest. However, there were striking similarities in the volumes of the major lung components across species, regardless of activity classification (Figure 2*a*; Table 3); neither percentage volume nor size-corrected absolute volume of the major lung components were significantly different for the 3 species.

At the TEM level, *M. splendens* was distinguished from the other study species with respect to the morphometric features of the plasma. This species had a significantly higher size-corrected plasma volume than *L. indistincta*, and a significantly higher percentage plasma volume than both *L. indistincta* and *H. neoxena*.

Palomeque et al. (1980) found that individual erythrocyte volume was consistent among passerine species, and also between passerine and nonpasserine birds. Thus, since the total volume of erythrocytes (V_e) was not significantly different across species, the number of erythrocytes in the pulmonary circulation should also be similar. For *M. splendens*, this erythrocyte number is contained within a proportionately larger plasma volume (V_p), so the stereologically derived haematocrit (Ht_s; Table 4) is significantly lower than that of the other 2 species.

Exchange tissue plasma volume is not, strictly speaking, an anatomical parameter of the lung but a component of the cardiovascular system. Carpenter (1975) found that avian species classified as 'strong fliers' (e.g. the pigeon, *Columba livia*) had a significantly higher venous haematocrit than those classified as 'weak fliers' (e.g. the turkey, *Meleagris gallopavo*). The low pulmonary haematocrit of *M. splendens* is similarly indicative of its classification as a 'low activity' passerine. Thus, while there were no differences between activity categories with regard to pulmonary volumetry, there is evidence of a relationship between pulmonary haematocrit and activity.

In conclusion, while there were no broad differences in pulmonary volumetry between passerines of different activity, the data suggest that there may be a cardiovascular basis for differences in metabolic activity. It remains to examine further other pulmonary parameters in the light of these findings, and also to explore in more detail the relationship between haematocrit, pulmonary stereology and metabolic activity.

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